SYMPOSIUM

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CENTRE OF EXCELLENCE FOR REPRODUCTIVE AND REGENERATIVE MEDICINE

NIKOLA ŠKREB SYMPOSIUM: NEW PLATFORMS IN DEVELOPMENTAL BIOLOGY - TOWARDS THE CLINICAL APPLICATION

ABSTRACTS

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Preface

We have dedicated Nikola Škreb Symposium to the memory of an outstanding scientist, the founder of the Zagreb School of Mammalian Embryology and above all to a teacher that inspired many to dedicate themselves to science. Being a medical doctor himself, Nikola Škreb understood that the basic biological research is the very fundament of every progress in medicine.

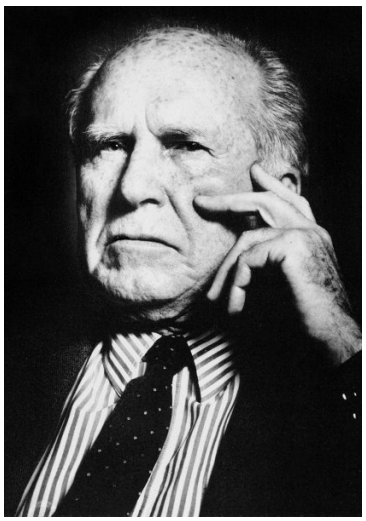
In 1986., when professor Škreb was supposed to retire, his students and collaborators D. Solter, I. Damjanov, D. Šerman and A. Švajger organized a memorable conference „Developmental Origins of Neoplasia" in Dubrovnik with participation of leading developmental biologists- from Spemann's collaborator Salome Gluecksohn-Waelsch to the future Nobel prize winner Martin Evans. This great occasion inspired us to organize Škreb Symposia at our School of Medicine in the memory of our teacher. In the first Škreb Symposium „Experimental Mammalian Embryology at the Turn of the Century (2003), where Davor Solter, Ivan Damjanov and Anton Švajger presented their research, we were honoured especially by participation of Anne McLaren and Andzej Tarkowski, the winners of that year's Japan Prize. Next Symposium was held in 2013. with Davor Solter, Barbara Knowles, Takashi Hiiragi, Rolf Kemler, Azim Surani, Anne Ferguson-Smith, Siniša Volarević, Dinko Mitrečić and younger members of the Department of Medical Biology.

The third Symposium in 2018. is one of the activities of the project Regenerative and Reproductive Medicine - Exploring New Platforms and Potentials, supported by the European Union through the European Regional Development Fund, Operational Programme Competitiveness and Cohesion, under grant agreement No. KK.01.1.1.01.0008, of the Center of Excellence on Reproductive and Regenerative Medicine, Research Unit on Reproduction and Development.

We hope that all participants will benefit from the interesting program.

Prof. dr. sc. Floriana Bulić-Jakuš
Prof. dr. sc. Davor Solter
Prof. dr. sc. Davor Ježek

Nikola Škreb biography



Nikola Škreb (1920-1993) was born in Zagreb. He graduated from the School of Medicine in 1948. and stayed on as a researcher and teacher at the Department of Biology until the very end of his life. Nikola Škreb did pioneering research in mammalian developmental biology and with his collaborators made major discoveries in that field. First, based on experiments with teratogenic agents (with Z. Frank, N. Bijelić, M. Mueller), he found out that „all or none" effect upon the embryo is lost with the critical phase of mesoderm formation, leading to congenital anomalies. Next, together with A. Švajger and B. Levak-Švajger, he discovered that the epiblast is the source of all three germ-layers. With D. Solter and I. Damjanov he found out that the mouse embryo gives rise to a malignant teratocarcinoma after transplantation under the kidney capsule of the adult animal. He also investigated differential gene activity during mammalian embryogenesis with D. Šerman and possibilities of in vitro cultivation at the critical phase of gastrulation with V. Crnek-Kunstelj, F. Bulić-Jakuš, M. Vlahović, G. Jurić-Lekić. Professor Nikola Škreb was the Head of Department from 1970. till 1993. and also the head of University Institute of Biology from 1962. till 1972. His research activities in mammalian embryology were supported by many domestic and several international USA and WHO grants. He was elected to the Yugoslav Academy of Sciences and Arts in Zagreb (1979), was the honorary member of the Croatian Medical Academy in Zagreb. He was awarded the „Ruđer Bošković Award" for science (1970), and the State Award for his Scientific Life Work (1979). Professor Škreb was elected the first president of European Developmental Biology Organization (EDBO) in 1978. and served as the president of the Croatian Natural Science Society.

Epigenetic Mechanisms Controlling Early Mammalian Development

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Epigenetic state of each cell is crucial in determining its gene expression and consequently its phenotype. Changes in the epigenetic state of the genome are essential for normal development. While gradual changes in epigenetic states occur throughout development there are two crucial moments when massive reprogramming of the genome takes place: transition from somatic cells to primordial germ cells and in zygote, following fertilization, when the germ cell epigenome transits to somatic epigenome state. During those transitions a general DNA demethylation takes places, however, there are several components of the genome e.g. imprinted genes that need to be protected. TRIM28 is one of the components of the protective mechanism and its absence during oogenesis leads to post-fertilization failure. Variations in failure phenotype suggest that, in absence of TRIM 28, epigenetic marks are randomly lost, and not replaced, from individual genes resulting in epigenetic chimerism. These results emphasize the importance of maintaining and protecting correct epigenetic state during mammalian development.

Role of Early-life Exposures on Epigenome and Cancer Susceptibility in Childhood and Adulthood

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There is growing evidence for the causal relationship between in utero and early- life exposures and increased risk of a wide range of human diseases, including cancer. Environmental agents and nutrients can produce important, stable, and, in some instances, transgenerational epigenetic alterations underlying changes in the phenotype, consistent with the 'Developmental Origins of Health and Disease' (DOHaD) hypothesis. Although causal relations have not been firmly established yet, the recent advance in epigenetics provides insight for the mechanisms of early life predisposition to child and adult disease risk. The levels and patterns of DNA methylation undergo dramatic changes during embryonic development, starting with a wave of a profound demethylation during the cleavage stage and followed by a widespread de novo methylation after embryo implantation stage. As these mechanisms operate in strictly defined stages of development, it is possible that these developmental periods (such as peri-conceptual period) represent particularly sensitive windows of vulnerability. Adaptive responses during in utero life may include epigenetic changes in different developmental pathways (such as production and expansion of somatic stem/progenitor cells, metabolic changes, and production of and sensitivity to hormones), a combination of which may alter normal development of tissues and organs. Our group has recently been involved in the studies aiming to test the hypothesis that epigenetic changes associated with childhood cancer risk and exposure to environmental/dietary/lifestyle factors during pregnancy can be identified in blood cells at birth, and that methylation changes can serve as sensitive biomarkers in primary and secondary prevention of childhood cancer. I will discuss recent evidence from our group and other laboratories indicating that epigenetic changes may be evident at birth and thus could constitute powerful mechanism-based biomarkers that could be exploited in disease prevention and epigenetics-based therapy.

Mammalian embryo/teratoma in vitro and therapeutic agents

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Embryos and tumors have many similarities because both depend on same developmental processes such as the growth, differentiation, and apoptosis. Therefore, agents used in treatment of tumors also act as teratogens on the mammalian embryo developing in utero. In our defined biological system in vitro, we omit confounding influence of the maternal organism and screen for teratogenic agents in an alternative to in vivo animal experiments. In this natural three dymensional biological system we are assesing the impact of various agents on gastrulation, the most critical phase of development. At the air-liquid interface, gastrulating rat embryo develops in an experimental teratoma-like tumor consisting of the main derivatives of the three germ-layers formed at that stage of development. Consequently, this system was also used to screen for therapeutic agents that may negatively influence tumor development. Our recent results with various hyperthermal regimes that negatively affected development of embryos/experimental teratomas, pointed to the pretreatment with an anti-heat shock therapy before the hyperthermal regime that completely destroys such a tumor. Recently, by the Fouriere transform infrared spectroscopy (FTIR) we discovered differences in metabolomes of spent media used during the 14-days in vitro culture with the antiepileptic valproate that also seem to reflect the developmental status of the embryo/teratoma. Results with a physical agent and an epigenetic drug speak in favor of our biological system in screening for teratogenic agents and for novel therapies of tumors.

Acknowledgments: This study was supported by Scientific Center of Excellence for Reproductive and Regenerative Medicine, Republic of Croatia, and by the European Union through the European Regional Development Fund, under grant agreement No. KK.01.1.1.01.0008, project „Reproductive and Regenerative Medicine - Exploring New Platforms and Potentials.

Limb Bud Development and Teratogenesis: in vivo/in vitro

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Modern genetic and functional analysis of the mammalian limb development have started to reveal fast changes in functions of development-driven genes and regulatory hierarchies during development. It is known that temporal and spatial interplay of the signaling centers instruct limb bud morphogenesis in a self- regulatory manner. This interplay can be interrupted by mutations and teratogens that disrupt progression of limb development most often by causing death of the early-specified progenitors, while their fate remains the same. Here the essentials of an in vitro 3D organ culture model of the limb bud development will be presented. Our system uses 13 and/or 14 days old limb buds which are microsurgically derived from rat embryos and cultured at an air-liquid interface from 3 to 14 days in serum-supplemented or chemically defined media. This system supports the viability of tissue, offering a variety of parameters to be monitored in the screening processes: cell proliferation, cell and tissue differentiation, cell death, size and the shape of limb explants and chondrogenesis. The example of how these parameters serve in the toxicity testing of chemicals (5azacytidine) or physical agents (temperature) with teratogenic potential will be shown. These results will be compared to in vivo research with an antioxidant in limb bud teratogenesis caused by 5azaC. Moreover, an overall methylation analysis in both models was done to explore the differences in DNA methylation due to the cultivation conditions and developmental stage. The results support the usage of this model to study processes of limb bud development.

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Testicular Germ Cell Tumors: From Mice to Men

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Testicular GERM cell tumors (TGCT) are rare malignancies, but in fact the most common in young men. Model-based predictions warn that Europe will see a further 25% increased burden, especially in Croatia. In line with the escalating incidence, and in support of future patient management, empowering research on mouse TGCT models seems to be crucial. Indeed, experimental mouse teratocarcinoma (emTCa), obtained by transplanting gastrulating egg-cylinder under the kidney capsule of syngeneic mouse, provides an outstanding system to study early TGCT development. In the frame of eight weeks development, emTCa grows steadily for six weeks after which it vigorously increases its weight. This period coincides with a peak in PCNA expression as well as a temporary SCGB3A1 tumor suppressor gene hypermethylation. Stemness genes OCT3/4 and NANOG show behaviour that is rather more complex. NANOG experiences a rise in DNA methylation immediately, after which remains stably hypomethylated in comparison to testis. NANOG mRNA level stabilizes in the sixth week and appears negatively correlated with emTCa growth in the eighth week. On the contrary, OCT3/4 shows constant rise in DNA methylation that overshadows that in testis, reaching a maximum again in the sixth week. This change in OCT3/4 DNA methylation corresponds to the mRNA level, and both negatively correlate with emTCa growth in the eighth week. By knocking down OCT3/4 and NANOG by short esiRNA treatment in vitro, expression of both genes was arrested and growth of tumours significantly inhibited. These data suggest that NANOG and OCT3/4 are indeed drivers of TGCT tumorigenesis and that their DNA methylation status could be exploited as a potential biomarker in identification of early TGCT.

Acknowledgments: This study was supported by Scientific Center of Excellence for Reproductive and Regenerative Medicine, Republic of Croatia, and by the European Union through the European Regional Development Fund, under grant agreement No. KK.01.1.1.01.0008, project „Reproductive and Regenerative Medicine - Exploring New Platforms and Potentials.

Authentic vs. Imitation: Trophoblast vs. Tumor

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Because of their ability to rapidly proliferate, migrate and invade into maternal tissue, trophoblast cells are responsible for the pseudo-malignant nature of the ingrowing placenta and in many respects resemble malignant tumor cells. However, unlike pathological malignant states, the proliferative, migratory and invasive potentials of the trophoblast are physiologically regulated as well as spatially and temporally limited to the uterus and early stage of pregnancy. Failure of these mechanisms is equally detrimental to reproduction as is the excessive trophoblast invasion, and only proper coordination between all these processes results in correct placental development and birth of a healthy child. Thus, insufficient invasiveness of the trophoblast may result in preeclampsia or in intrauterine growth restriction (IUGR), whereas inordinate invasion can lead to abnormal attachment of the placenta with uterine tissue layers (myometrium and perimetrium), resulting in placenta accreta, placenta increta or placenta percreta. In addition, uncontrolled trophoblast invasion may lead to a range of gestational trophoblastic diseases (GTD), including gestational trophoblastic neoplasia, as well as development of reproductive and somatic tumors with pronounced trophoblastic component. To address some of the questions regarding normal vs. pathological trophoblast behavior as well as the development of ovarian neoplasms, we started studying the signaling pathways important for normal embryonal development, first the Wnt signaling pathway and, more recently, the Hedgehog signaling pathway. We shall present our own results on analysis of invasive processes in trophoblast/placenta and tumor tissues, obtained as a part of the research at the Center of Excellence for Regenerative and Reproductive Medicine from 2014 until today.

Acknowledgments: This study was supported by Scientific Center of Excellence for Reproductive and Regenerative Medicine, Republic of Croatia, and by the European Union through the European Regional Development Fund, under grant agreement No. KK.01.1.1.01.0008, project „Reproductive and Regenerative Medicine - Exploring New Platforms and Potentials.

In Vivo Visualization of Developmental Markers in Evaluation of Mouse Brain Repair After Ischemic Lesion

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Brain development is a dynamic period when neurons are born, differentiated, start their functional activities or being shed by process of apoptosis. In case of brain injury, as in stroke, it would be advantageous to invoke the developmental processes in order to repair the brain damage. However, the repair abilities of the brain are rather limited, hence the stroke is number one cause of disabilities in the developed countries. In vivo imaging in small animals is an important method to monitor and evaluate the processes which occur after ischemic injury achieved by middle cerebral artery occlusion (MCAO), being model of ischemic stroke in humans. The imaging modalities used in our imaging platform (GlowLab) are magnetic resonance imaging (MRI) and optical bioluminescence imaging (BLI). MRI provides insights in structural brain changes, while BLI visualises the expression of genetic markers in the brain providing molecular aspects of the brain response. For BLI we used a developmentally important marker Gap43, which is involved in axonal outgrowth, both in development and after brain injury. Our specific interest was interplay between neuroinflammation and brain repair. The inflammation was modulated by loss-of-function of Tlr2, hence Tlr2-mediated inflammation was missing in these animals. In vivo BLI of Gap43-expression showed the increased repair in the Tlr2-deficient mice. Still, the proper measurements of BLI-signal required normalization by MRI-measured stroke volume. The in vivo imaging was confirmed by Western-blot analysis of brain samples. The obtained results highlight the benefits of multimodal approach, and confirm the usefulness of developmental markers in evaluation of the brain repair.

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Multimodal imaging was done at Laboratory for Regenerative Neuroscience - GlowLab, University of Zagreb School of Medicine.

Influence of Hypoxia on Behavior of Developing Neural Precursors

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To understand mechanisms which influence the fate of developing neural precursors is important because of at least two major reasons: their differentiation occurs during normal (and abnormal) neural tissue development and moreover, it brings direct implications in potential treatment of nervous tissue by using transplantation of neural precursors. One of the important questions, still bringing controversies is: If we transplant stem cells in the brain affected by hypoxia – do we want them to follow their usual differentiation path? Or do we want something specific for hypoxic environment? In this work we describe application of stem cell in experiments which aim to elucidate both pathological and regenerative chain of events caused by lack of oxygen in the nervous tissue. Here we present our results showing that transplantation of stem cells in the brain affected by ischemia significantly influences expression of genes linked to cell death. This explains at least part of the beneficial effects observed in preclinical and clinical trials testing stem cell - based therapies for perinatal ischemia and stroke. Application of neural stem cells, automated microscope for in vitro cell tracking and specialized software allowed us to observe and quantify set of parameters occurring during neurogenesis in hypoxic conditions. Discovery of specific molecular pathways involved in tissue response to hypoxia/ischemia represents an important milestone in the current research activities which aim to propose new therapeutic targets for nervous tissue damaged by the lack of oxygen.

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Histone Variants H3 Essential Role in the Maintenance and Reprogramming of the Epigenetic Memory Carried by H3K27me3

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The epigenetic mark H3K27me3 controls spatial and temporal patterning throughout the flowering plant life cycle. The histone variant H3.1 is essential for the propagation of H3K27me3 (Jiang and Berger, Science, 2017). Whether this mark is extensively reprogrammed at any point in development has remained unclear. We show that H3K27me3 is selectively erased from sperm chromatin in Arabidopsis through a multi-layered mechanism, which includes the deposition of a sperm-specific histone H3 variant that is immune to methylation at lysine 27. Resetting of H3K27me3 also coincides with selective types of deposition of H3K4me3 in a manner that potentiates expression in mature sperm and at distinct phases of development after fertilization. In stark contrast to the global resetting of epigenetic marks in mammals, plants have evolved mechanism to selectively reprogram H3K27me3. This mechanism acts as an epigenetic timer to control gene expression during plant development.

The New Biomedicine

Barbara B. Knowles

Professor Emerituss, The Jackson Laboratory, USA

True success is not in learning but in the application of this knowledge to the benefit of mankind. The acquisition of basic scientific knowledge by scientists working in genetics, developmental- cell- and molecular-biology, immunology and virology throughout the 20th century has led to its application in clinical problems in the 21st century. The technology to repair genetic defects in pluripotent cells and to differentiate these cells into progenitors of healthy tissue is almost within our power. However, the application of this knowledge to living organisms using cells that can mutate and selectively evolve makes clinical application tricky, whilst social and ethical issues arising from the use of this new technology requires thought. Attention to trends in basic science research should make stem cell therapy applicable to all.

Human Testis: From Development to Clinics

Davor Ježek

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The seminiferous epithelium consists of supporting somatic Sertoli cells and spermatogenic cells. Spermatogenic cells are engulfed by the Sertoli cells cytoplasm & membrane. The unique feature of the neighbouring Sertoli cells are tight junctions positioned at the end of their basolateral cell processes, thus dividing seminifeorus epithelium into two compartments: basal (with spermatogonia) and apical (adluminal or meiotic, with haploid spermatogenic cells predestined to became spermatozoa. During the development of the indifferent gonad, under the influence of Sry, the vast majority of pre-Sertoli cells are thought to migrate inside the mesenchyme of the genital ridge from the surface coelomic epithelium. Namely, the coelomic subpopulations of cells that express steroidogenic factor-1 (Sf-1) are picked by delaminating signals of the underlying mesenchyme of the genital ridge. They differentiate into pre-Sertoli cells (that express fibroblast growth factor 9 /Fgf9/ and Sox 9 /Sry –related HMG box-9/) and become the chief organisers of sex cords. The expression of Sf1 is maintained in early Sertoli cells and Leydig cells. Under the influence of Sf1, Sertoli cells produce anti-Müllerian hormone (Amh), whereas Leydig cells produce foetal testosterone. Thus, Sertoli cells are crucial for the normal function of the adult testis and the development of the "male" genital ridge. The normal onset of spermatogenesis & spermiogenesis after the puberty enables the production of spermatozoa. Therefore, the testis can be a valuable source of male gametes during microsurgical techniques such as testicular sperm extraction (TESE), employed for the treatment of male infertility.

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Using an IV Spermatogenesis Platform to Model Male Factor Infertility and to Develop Novel Stem Cell-based therapies

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Spermatogenesis is a critical process that ensures transmission of the paternal genome to offspring during fertilization. However, there are a number of external factors that can affect spermatogenesis even resulting in sterility. Recent studies have indicated that sperm parameters in Western males have sharply declined by as much as 50% over the last 40+ years. While the causes of this sharp decline are unknown, the overall data indicate that infertility rates in Western men may be steadily increasing. To date, nearly 15% of couples worldwide experience infertility issues, with male contributing factors playing a role in over 30% of these cases. With infertility rates in men sharply rising, investigations need to be conducted into a) what factors are contributing to this rise in infertility and b) what therapies can be derived to treat male factor infertility. Here, we utilize a novel human in vitro model of spermatogenesis to examine environmental impacts on male fertility. Additionally, we use a non-human primate in vitro model of spermatogenesis to highlight a novel therapeutic approach for treating male infertility in cases where men fail to produce functional gametes for IVF.

Early Lung Epithelial Progenitors Originating From Human Pluripotent Stem Cells

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Currently, it is a challenge to obtain sufficient numbers of primary lung epithelial progenitor cells that could possibly be used for therapy and/or tissue engineering applications. Here we describe the cells differentiated in vitro from human embryonic stem cells (hESC) that can be propagated for long-term in culture and most likely represent equivalent of early lung progenitors (ELEP) occurring in development. We have shown that these cells can be maintained in culture for a minimum of 65 passages without losing their key characteristics. ELEP maintain their population doubling time at an average of 26.5 hours and the activity of their telomerase holds at about 50% of that typical for undifferentiated hESC. ELEP express high levels of anterior foregut marker SOX2 (also typical for self- renewing cells), marker of definitive endoderm SOX17, and marker of early lung epithelial lineage, thyroid transcription factor-1. As found by transmission EM, ELEP also possess morphological features of cells differentiating towards airway epithelia, multivesicular and lamellar bodies. When induced to terminally differentiate, ELEP increase levels of FOXJ1 (ciliated cells), pro-surfactant protein B (alveolar epithelial cells), Club cell specific protein (Club cells), aquaporin A (type I pneumocytes), and surfactant proteins A and C (type I pneumocytes and Club cells). Under 3D conditions, differentiating ELEP then develop morphologies of alveolar- and airway-like structures.

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Novel Technology for Regeneration of Bone Defects

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We developed a novel Autologous Bone Graft Substitute (ABGS) that is composed of recombinant human BMP6 (rhBMP6) and Autologous Blood Coagulum (ABC). ABGS uses low doses of BMP6 as compared to BMP2
(InFuse®) and BMP7 (Ossigraft®) and devoid of animal derived collagen, thus elicits little or no inflammation and immune responses. We performed safety and efficacy preclinical studies using Rabbit Ulna Segmental Defect model and Posterolateral Lumbar Spine Fusion models in Rabbits and Sheep. ABGS was examined by alone and in combination with allograft or synthetic ceramics, Compression Resistance Matrices (CRM) in spine fusion models. The results demonstrated that ABGS induced a robust bone formation that resulted in restoration of segmental defects and complete achieved fusion in between two transverse processes at bilateral lumbar spine sites. The successful preclinical studies allowed an approval to examine the safety and efficacy of ABGS in Phase I/II clinical studies in patients with a Distal Radius Fracture (DRF) and High Tibial Osteotomy (HTO). ABGS is fabricated to render a flexible and injectable implant ensuring the ease of use. In DRF studies, three groups of patients enrolled: standard of care, ABC alone and ABGS (ABC+rhBMP6). The ABGR device was formulated from 1 mL of ABC and 250 µg of rhBMP6, while placebo was formed from 1 mL of ABC. No measurable plasma amounts of rhBMP6 were detected after 5 min to 7 days, or BMP6 antibody after 13 weeks. In addition, no serious adverse effects (SAE) have been observed, including swelling, redness of the skin, edema, distant soft tissue ossification, pain or systemic side effects. The use of a small dose of rhBMP6 in ABC minimized the risk of side effects and antibody production as reported for BMP2 and BMP7 in such safety studies using DRF patients. In patients with HTO, the weight bearing part of the knee was shifted from overloaded knee compartment to realign the knee pressure. A randomized, double blind, placebo controlled trial was conducted in two stages in 20 patients treated with 10 ml ABC containing placebo or 1 mg rhBMP6. Phase I included 6 patients and in Phase II the remaining 16 patients were enrolled. No SAEs have been reported, and at 24 weeks of radiological follow up there were different patterns of bone healing within the osteotomy wedges observed. The radiographic scoring of x-ray and CT data showed an accelerated osteotomy healing as compared to placebo treated patients. Our clinical study is the first evidence that BMP6 is safe and effective in the bone metaphyseal compartment when directly administered inside the trabecular bone area as opposed to reported unwanted safety issues in BMP2 and BMP7 when used at trabecular surfaces. Evaluation of ABGS with a CRM is in progress using a randomized Phase II Posterolateral Lumbar Interbody Fusion (PLIF) clinical study.

Autism Spectrum Disorder in Patients with Rare Diseases

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Autism Spectrum Disorder (ASD) is heterogeneous group of neurodevelopmental disorders defined by impaired social-communication and presence of restricted and repetitive patterns of behavior. ASD has a strong genetic basis indicated by higher reccurence risk within affected families as well as the co-occurence with rare genetic syndromes. Objective of our research was clinical and molecular analysis of ASD patients in order to identify rare disorders associated with copy number variants (CNVs). Study included 110 children with psychiatric diagnosis of ASD. Patient's samples were analyzed using chromosomal microarray from Jan 2016 until Jan 2018. CMA analysis detected CNVs in 21 of 110 patients studied. Pathogenic CNVs were found in 13 patients (Group A) while in additional 8 patients we detected variants of unknown significance (VUS) (Group B). Genetic changes found among patients in Group A included: 1p36 deletion (1), 2q33.1 deletion (1), 8p23.1 microdeletion (1), 15q11.2 microdeletion (2), 15q11-q13 duplication (1), 15q13.3 microdeletion (1), 17p11.2 deletion (1), 22q11.21 microduplication (1) and 22q13.3 deletion (1). We also found small deletions associated with autism that affected region 2q21.1 (1) and genes CNTNAP2 (1), NRXN1 (1). All patients in Group B had duplications that ranged from 131Kb to 1.6Mb and affected genes/regions are known to be involved in neuronal development. All these CNVs were inherited from healthy parents and their clinical significance is uncertain. Detailed clinical and diagnostic assessment should be performed in all patients presenting with ASD. CMA proved to be useful diagnostic tool in diagnosing rare genetic conditions in autistic patients.

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Epigenetic Biomarkers of Prostate Cancer (epiPro)

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This project includes two interconnected goals. The first is to establish a new Scientific Group for Research on Epigenetic Biomarkers, epiMark. Scientists gathered through epiMark are already active in studying male reproductive health and disease, therefore will contribute to the next general goal: studying potential epigenetic biomarkers (EB) in liquid biopsies of patients diagnosed with prostate cancer (PCa). PCa is second in incidence and third cause of cancer deaths in Croatia, with incidence rates steadily rising. Contrarily, Croatia records a fall in PCa research. With PSA being quite sensitive biomarker, but not specific for PCa, there is a need for developing new diagnostic tools. Our aim is to investigate microRNA and DNA methylation which may serve as potential EB for PCa risk and disease progression, and tools for differentiating PCa and benign prostate hyperplasia (BHP). According to recent findings, we chose a set of miRNAs and gene promoter regions believed to be associated with PC development and progression. The study will be conducted on volunteers diagnosed with PCa and BHP within routine at Urology departments of UHC Zagreb and UHC Sestre milosrdnice. In seminal fluid and blood, profiles of genes DNA methylation within cfDNA will be identified by pyrosequencing, while expression of miRNA by qPCR and ddPCR. Epigenetic analysis of gene expression will be performed on patient cancer tissues as well. By comprehensible statistical analysis, we expect to identify potential EB of PCa in liquid biopsies

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Osteoclast Progenitors Expressing CCR2 are Expanded in Collagen-induced Arthritis and May Be Involved in Bone Resorption Intensity

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Osteoclast progenitors (OCP) originate from the myeloid lineage and mature to osteoclasts, specialized bone resorbing cells, which mediate bone destruction under inflammatory conditions in rheumatoid arthritis (RA). We investigated frequencies and osteoclastogenic potential of OCP subpopulations in bone marrow of affected joints (BM) in collagen-induced arthritis (CIA), a mouse RA model. CIA development in C57BL/6 and DBA mice immunized with chicken type II collagen was confirmed by micro-CT, histology, and serum CTX levels. BM cells were immunophenotyped for hematopoietic markers and chemokine receptor expression. Sorted OCP subsets were cultured with M-CSF/RANKL and assessed for TRAP+ osteoclast number, proliferative response by CFSE, and in vitro migration potential using Transwell system with chemotactic gradient. Mice developing CIA were treated with methotrexate (2mg/kg) and CCR2 receptor antagonist (CRA) (4mg/kg) to assess effects on OCP frequency. Frequency of CD45+B220-CD3-NK1.1-Ly6G-CD11b–/loCD115+ OCPs was significantly increased in CIA (54% vs. 26% in control), with specific expansion of the CCR2+ subset. Regarding the CCR2 expression level, the CCR2lo subset underwent more divisions and generated multinucleated TRAP+ osteoclasts more efficiently, whereas the CCR2hi subset generated osteoclasts only when cultured at high density. OCPs from CIA mice demonstrated significantly enhanced migration toward CCL2 gradient. Our preliminary results indicate that a combined methotrexate/CRA treatment may affect the frequency of OCPs. OCP subpopulation expressing CCR2 may contribute to increased homing of OCPs to bone surfaces of inflamed joints. Therefore, inhibition of CCL2/CCR2 signaling presents a new therapeutic approach to reduce osteoclast activity in arthritis.

Impact of Endocrine Disruptors on Androgen and Estrogen Receptors During Intrauterine Brain Development

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Transplacental exposure to endocrine disruptors (EDs) may impair concentration, motor function, and language development, reduce IQ, and possibly induce autism. In addition to epigenetic modifications of DNA and noncoding RNAs, diverse exposure profiles and mixtures of EDs, such as heavy metals, plasticizers, coupled with estrogen (ER) and androgen receptors' (AR) may trigger in the developing brain adverse pathways at much lower toxic levels than conventional. Disturbance of estrogen/testosterone balance during early brain development may cause discordance of mitotic dynamic and maturation sequence during neuron migration crucial for building of complex brain architecture. Subplate zone as a major site of synaptogenesis is a transient part of the human brain whose peak of activity is seen at 22-24 gestational weeks (GW). The expression of aromatase in subplate zone starts around 16thGW but there is no data available on its possible alterations by EDs. As majority of environmental EDs are xenoestrogens it is important to stress that ER-alpha is detected in the cortex as early as during the 9thGW, with high expression in proliferating zones and the cortical plate. In an animal model is has been shown that bisphenol A disturbs levels of ER-alpha, accelerate neuronal differentiation and migration. Additionally, aromatase inhibitor cotinin increases testosterone levels and disturbs AR levels, which have crucial roles in hippocampal neurogenesis and corpus callosum development. It is hoped that prospective data integration by Systems Biology Graphical Notation using deep learning artificial intelligence could serve for real time investigation of complex EDs effects during intrauterine brain development.

Molecular Analysis of RB1 Gene Expression Dynamics During the Pluripotency Transition in the Mammalian Embryo

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RB1, the retinoblastoma susceptibility gene is an important regulator of cell proliferation, apoptosis and differentiation. The RB protein shows an important function in the cell cycle by controlling the G1/S phase transition. In its hypophosphorylated form, RB is active and supresses the cell cycle progression, as it inactivates transcription factors (TFs), mostly from the E2F family. In G1, the formation of cyclinD/CDK4/6 complexes hyperphosphorylates the RB, causing its disability to bind E2F family members, which then leads to the progression to the S phase. Embryogenesis provides a system to investigate the roles of RB family proteins at the interface between proliferation and differentiation. The most compelling evidence of the importance of RB in cellular differentiation comes from studies of RB1 knockout mice, where the disruptions of the RB1 gene cause death by day 14 of gestation, associated with defects in the development of the hematopoietic system and central nervous system. Studies on immunohistochemical expression in the mouse and adult human showed a cell- specific heterogenous pattern in various tissues. RB is expressed in differentiated cells and is absent from cells that still proliferate (e.g. mouse retina, stratified squamous epithelium, and gut epithelium of the mouse, adult human cervical epithelium of the uterus). Methylation patterns for the retinoblastoma gene (RB1) in single human blastocysts showed 40%-60% methylation, indicating that a methylation imprint was present. Its hypermethylation also occurs in tumors, associated with RB1 gene disfunction. By using modern molecular methods (pyrosequencing, ddPCR) and immunohistochemistry, we shall try to determine yet unknown RB gene methylation and expression changes in transition from the pluripotency to the differentiated state in mammalian embryos.

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Preparation Protocols for Mouse Ischemic Brain Lesion Evaluation: Multimodal Approach

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Multimodal imaging and volumetric analysis of the mouse ischemic brain are used to evaluate potential neuroprotective therapies and to establish new therapeutical approaches during preclinical trials. When using several imaging techniques we can obtain more information and reduce the number of experimental animals, simultaneously allowing macroscopic and microscopic 3D evaluation of the ischemic lesion evolution. C57Bl/6 albino mice underwent 60-minute cerebral ischemia induced by the middle cerebral artery occlusion method. The first two groups of animals underwent different fixation protocols: the Evaporation-of- Organic-Solvent (EOS) dehydration method for synchrotron imaging and hydration for MR imaging. The other two groups underwent different contrast agent staining procedures using phosphotungstic acid or non-ionic iohexol monomer staining. Different fixation protocols influenced MRI quality and contrast of the visualized lesion, which was easily delineated by SRμCT imaging when the EOS method was applied. Contrast agents enabled better ischemic lesion visualization only for synchrotron and micro-CT imaging. Volumetric analysis of the ischemic lesion and brain oedema was clearly influenced by the preparation methods used, which differently affected the lesioned area and the healthy tissue, producing a huge variability in the measured volumetric values. MRI enables good gross morphology visualization of the brain tissue, while micro-CT and SRμCT provide high resolution neuroarchitectonic depiction.

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The MRI scans were performed at the Laboratory for Regenerative Neuroscience – GlowLab, School of Medicine University of Zagreb, Croatia.

Analysis of c-KIT Expression in Nonseminomatous Testicular Germ Cell Tumors

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Incidence of testicular germ cell tumors (TGCT) has been continuously rising (1- 5% per year). (In Croatia the incidence of TGCT is increasing at the highest rate in the world. Croatia also has a high rate of mortality compared to other countries. Germ cell neoplasia in situ (GCNIS) is a precursor lesion of TGCT's which makes up to 95% of all testicular tumors. TGCT are divided into pure seminomatous and non-seminomatous tumors. GCNIS is considered to be driven by an interplay of genetic, epigenetic and micro-environmental factors that lead up to an arrest of gonocyte differentiation. GCNIS is reprogrammed in the development of non- seminomas into embryonal carcinoma (EC) cells, which make up the pluripotent core of the non-seminomas. Currently used biomarkers for testicular cancer lack both specificity and sensitivity, and are used primarily as accessories in diagnostics. A deeper look into the molecular signature of nonseminomatous tumors is needed, due to their often times aggressive progression and cisplatin resistance. The aim of this study is the analysis of protein expression in nonseminomatous tissue as a potential biomarker of nonseminoma development and progression. Formalin-fixed paraffin-embedded tissue from 43 nonseminomatous testicular tumors from the Ljudevit Jurak Pathology and Cytology Department Archive, from the University Clinical Hospital Centre Sestre Milosrdnice, were used for immunohistochemical detection of c-KIT. Slides were analyzed semi-quantitatively, at the area of strongest reaction, by pathologist, on a scale from 0-3, depending on the percentage of reactive cells. The data was analyzed in GraphPad Prism using the Mann-Whitney test. The results have shown c-KIT staining in non-seminoma tissue to be comparable to healthy testis tissue. The intensity and positivity of c-KIT staining in non-seminoma is either fully absent, or if a "weak" signal is present it is mostly in the teratoma component. Germ cell neoplasia in situ (GCNIS) is c-KIT positive. The difference in c-KIT protein expression between GCNIS and teratoma versus other non-seminoma and healthy testis tissue opens ia question on the role of c-KIT in non-seminoma development and differentiation.

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Molecular Analysis of Leydig Cells in Patients With Non-obstructive Azoospermia (NOA)

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Non-obstructive azoospermia (NOA) is a severe diagnosis of male infertility characterized by the complete absence of sperm in the ejaculate with different level of spermatogenesis abnormalities. Various histological anomalies have been detected in the testicular tissue biopsies of NOA patients such as deterioration of seminiferous tubules and belonging cells (germ cells, Sertoli cells) together with morphological changes of Leydig cells. Leydig cells are androgen producing cells located in the interstitial space, adjacent to seminiferous tubules and near blood capillaries. It has been demonstrated that Leydig cells within the same NOA patient may be considered mosaic. Part of the Leydig cell population may increase in size (hypertrophy) and number (hyperplasia) with 15 or more cells clustered together, another portion may contain extremely vacuolated cytoplasm while some Leydig cells produce testosterone in excess. Therefore, the aim of our research will be to elucidate the molecular profile behind Leydig cell morphological variations in NOA testes with normal testosterone production. Leydig cells will be analyzed by the presence of their hypertrophy, hyperplasia and vacuolization. Workflow will include isolation of Leydig cells with laser microdissection system, cell lysis and isolation of nucleic acids. Expression of genes implicated in steroidogenesis, synthesis of male androgens, and proliferation will be analyzed on single cell level with ddPCR and/or qPCR. Single-cell sequencing methods will be also performed to study DNA methylation. Protein expression will be analyzed by immunohistochemistry. We expect to define set of genes which will explain the Leydig cell dysfunction and morphological changes in NOA patients.

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Genetic Counselling Unit-model and Experience of University Hospital for Tumours Sestre milosrdnice

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Genetic counselling unit exists as a separate functional unit in University hospital for tumours from the year 2015. It is based on cooperation and teamwork of experts of different profiles: medical geneticist, surgeons, oncologists, molecular geneticists, specialized nurses and psychologists. The aim is to recognize and counsel patients with family and personal history indicative of the hereditary cancer syndrome. Most of the patients are those suspected to have hereditary breast and ovarian cancer syndrome, although there are also patients with familial adenomatous polyposis, Lynch syndrome (hereditary non-polyposis colorectal cancer syndrome), neurofibromatosis and few other very rare hereditary cancer syndromes (e.g. von Hippel-Lindau disease, Li Fraumeni). Clinical signs pointing to the possibility of pathogenic mutation in some of the predisposing genes (early age of onset, typical histology, multiple tumours...), along with confirmed family history are the base of selection. Patients with such disease characteristics are referred to a genetic counselling centre with the assessment of indication for further genetic testing. Genetic testing of hereditary breast and ovarian cancer syndrome (BRCA1 and BRCA2 genes), as the most commonly identified hereditary cancer, is now available and covered by Croatian health Insurance Fund through the programme of genetic counselling in an authorized institution. The analysis is performed by next generation sequencing technology coupled with quantitative polymerase chain reaction (qPCR) for detection of large deletions and duplications. The positive result is confirmed by Sanger sequencing. Genetic testing is performed in a Laboratory for hereditary cancer and Laboratory for advanced genomics at Rudjer Boskovic Institute, Zagreb. Patient receives genetic counselling before and after genetic testing. Primary and secondary prevention measures that could be undertaken after the results of hereditary breast cancer genetic testing in a high risk population contribute to savings in the health care system and the better quality of life in a group of high risk individuals.

Involvement of Key Modulators of Wnt Signaling, sFRP1 and sFRP3, Across Malignancy Grades of Human Astrocytoma

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SFRP gene family code for important regulatory proteins of Wnt signaling usually limiting pathway's activity. We investigated the involvement of SFRP1 and SFRP3 in astrocytomas of different malignancy grades in order to better understand their behavior in tumor progression. The expression was analyzed by immunohistochemistry, digital scanning and image analysis. Promoter hypermethylation was examined with methylation-specific polymerase-chain- reaction. Our results demonstrate that hypermethylation of SFRP1 promoter was progressively rising in astrocytoma grades with the highest distribution in glioblastoma (P=0.042). Furthermore, cases with methylated promoter expressed significantly less SFRP1 than unmethylated ones (P=0.031). Pathway's indicators of oncogenic activity, beta-catenin, LEF1 and TCF1, were also explored. Glioblastomas with unmethylated SFRP1 promoter had significantly less beta- catenin (P=0.033), while strong expression of both LEF1 and TCF1 was associated to higher astrocytoma grades (P=0.006). The results on SFRP3 involvement were not so straightforward demonstrating different behavior in subcellular compartments. Stronger nuclear expression values were associated with lower astrocytomas grades (P=0.028) as compared with astrocytoma grades III and IV. Contrary, stronger cytoplasmic expression levels were higher in the astrocytoma III and IV group than in the astrocytoma I and II group (P=0.048). Our findings show that SFRP1 gene behaves as a classical tumor suppressor and was significantly epigenetically silenced in glioblastomas as compared to low astrocytoma grades, suggesting its lack allows progression. SFRP3 protein demonstrated dual behavior as an antagonist of Wnt signaling when found in the nucleus, whereas when located in the cytoplasm as an agonist of Wnt signaling promoting invasive behavior.

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Expression of KIT Gene in Testicular Seminoma

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Testicular neoplasia represents only 1% of all neoplasia, but it affects young male population. Approximately 95 % of all testicular cancers are germ cell tumors. There are two groups of TGCT, seminoma and non-seminoma. Compared to non– seminoma, seminoma is hypomethylated. The hypomethylated genome is unstable and more exposed to mutations what brings to the emergence of tumor cells. Hypomethylated genes show higher expression rate than other genes. Consequently, the gene expression pattern between tumor and healthy tissue differs. Gene expression on the protein level is used for diagnosis of tumors. OCT ¾ is a gene which is already used as a specific marker for the diagnosis of seminoma, but new markers could contribute to diagnostic protocols and patient management. Archive seminoma slices were collected. Immunohistochemical staining was done manually, on 5µm archive tissue sections in the Laboratory for Epigenetics and Molecular Medicine. To visualize our genes of interest, we used specific antibodies. Morphometric analysis of gene expression at the protein level was performed at University Hospital Sestre Milosrdnice Department of Clinical Pathology Ljudevit Jurak. Differential gene expression in tumor tissue and in healthy tissue was observed. Analyzing and quantifying expression of genes at the protein level can contribute to the development of new histopathological markers for seminoma in blood and semen.

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Clinically Relevant Copy Number Variants in Children With Congenital Anomalies

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Congenital anomalies (CA) encompass a wide array of structural and functional abnormalities that can occur in isolation, or can be associated with other anomalies and/or developmental problems. When CA are associated with additional clinical features (ACF), they often result from genetic defects or teratogenic factors. Copy number variants (CNVs) are among important causes of genetic syndromes associated with CA. Chromosomal microarray (CMA) is used as the first test to detect the CNVs in this category of patients. The aim of the study was to determine clinically relevant CNVs in 233 unrelated subjects analysed in the period between January 2016 and December 2017 in Children's University Hospital Zagreb because of the presence of one or more CA and ACF. In addition, we have analysed a group of 70 patients with isolated congenital heart defects (CHD). All clinically relevant CNVs were compared with those reported in public genomic databases, and their clinical significance was evaluated. CNVs were detected in 96 (41.2%) patients. Pathogenic variants were detected in 76 (79.2%) and VOUS in 20 (22.3%) subjects. Clinically relevant CNVs were slightly more frequent in patients with multiple CA (48.0%) than in patients with single CA (35.9%) (p=0.0604). In the group of patients with isolated CHD two pathogenic deletions and three VOUS were detected resulting in the diagnostic yield of 7.1%. CMA has proven to be a valuable test in patients that in addition to one or more CA have developmental delay/intellectual disability, behavioural problems and/or dysmorphia, establishing the diagnosis in 41.2% of patients.

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Epigenetic Research of Developmental Disorders/congenital Anomalies

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Intellectual disability/Developmental delays (ID/DD) are present in 2-3% of children and the worldwide population prevalence of autism spectrum disorder (ASD) is about 1%. Congenital anomalies (CA) are the leading cause of stillbirth and infant mortality and an important contributor to childhood morbidity. Over 130,000 children with CA are born in Europe each year. Their physical, mental and social handicaps are a significant burden on the healthcare, social and educational services. Therefore, all these disorders are an important public health problem and a major diagnostic challenge, due to their complex etiology. To promote the research in this area, the project "Reproductive and Regenerative Medicine - Exploration of New Platforms and Potentials" of the Center of Excellence for Reproductive and Regenerative Medicine (CERRM), has set "Epigenetic Research of Genetic Disorders/Congenital Anomalies" as one of its topics. The target group patients consists of children with ID/DD, ASD, CA and rare neurodevelopmental disorders of unknown aetiology. The project will investigate the underlying genetic and epigenetic mechanisms, with special reference to genotype-phenotype correlation, opening up new ways of treatment and prevention strategies, and application of genomics and epigenomics in the clinical practice. It is expected that the study will identify new epigenetic markers, candidate genes and pathogenic variants associated with conditions under study, establishing accurate genetic diagnosis that will allow genetic counseling, prenatal and preimplantation diagnosis and cascade screening of family members at risk. The research could open the way of finding new therapeutic options based on the epigenetic modification of targeted genome regions.

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Comparative Study of Human and Experimental Mouse Teratocarcinoma

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Teratocarcinoma (TCa) is a type of mixed testicular germ cell tumor (TGCT) composed of teratoma and embryonal carcinoma. In 1970s Solter, Damjanov and Škreb discovered that TCa could be produced by transplanting gastrulating mouse embryos underneath a kidney capsule of a syngeneic animal. TCa was identified by classical histological analysis and now this experimental mouse teratocarcinoma model is considered as suitable for studying TGCT biology. Molecular systematic analysis and comparison of human and mouse model TCa have not been performed. The purpose of this research is to compare proliferative and apoptotic activity in human and mouse experimental TCa. For immunohistochemical detection of PCNA and Caspase-3 expression, 20 human testicular teratocarcinoma and 14 experimental mouse teratocarcinoma paraffin- embedded tissues were used. Slides were semi-quantitatively analyzed and results were categorised regarding percentage of IHC-positively stained cells as 0 (0%),1 (<10%), 2 (10-50%) and 3 (>50%). Differences in rates of proliferation and apoptosis between human and mouse TCa were statistically significant. Most of experimental mouse TCa (64%) and just 30% human TCa showed >50% PCNA-positively stained cells. With respect to Caspase-3 staining, mouse TCa samples were similarly distributed among categories 1, 2 and 3 while 70% of human TCa belonged to category 1. Analysis of proliferation and apoptosis based on percentage of PCNA and Caspase-3 positively stained cells statistically significant differ between human and mouse experimental TCa. This result may be due to small and unequal number of samples. Further analysis should be done and we are planing to conduct Western blot analysis of PCNA and Caspase-3.

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A New Non – Invasive Model of Perinatal Hypoxic Brain Lesion Causes Lifelong Changes in Perineuronal Nets and the Learning Behavior in Rats

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Aiming to investigate changes in neuronal connectivity after perinatal hypoxic brain lesion, we were analyzed perineuronal nets and cognitive behavior in a new non-invasive model of brain injury in rats. Nineteen Wistar Han® (RccHan®:WIST) rats, (9 females and 10 males) were randomly divided into hypoxic and control group on postnatal day 1 (P1) when hypoxia was induced in a warm (≈ 25°C) hypobaric chamber (Atm 350mmHg, pO273mmHg) during 2 hours, while controls were kept in normal housing conditions. Behavioral tests were performed at P30 and P70 using the open field, hole board, social choice, and T-maze tests. Samples of brain tissue from adult animals (P105) were used for histochemical examination of the cytoarchitectonics (Nissl staining), interneurons (parvalbumin immunohistochemistry) and perineuronal nets (Wisteria floribunda agglutinin, histochemistry). After mild perinatal hypoxic brain injury, cerebral cytoarchitectonics, as well as the laminar and structural organization of the telencephalon were preserved. However, changes in morphology, number, and distribution of the parvalbumin-immunoreactive neurons and perineuronal nets, distinct in different regions of the telencephalon, were observed. Moreover, motor and socialization patterns were preserved, while treated animals showed significantly impaired learning behavior. In conclusion, a short-term perinatal hypoxic brain injury in rats leads to disturbances in brain connectivity related to cognitive processes consistent through development and adulthood. Further characterization and evaluation of this brain injury model, on molecular, cytological and connectivity levels, is needed to disclose developmental disturbances caused by provoked hypoxia that are not compensated during development and lead to cognitive deficits still present in adult rats.

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The Role of Semaphorin5A Protein During Brain Development in Rodents

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The Semaphorin-5A (Sema5A) is a transmembrane protein that has been shown to have a role as bifunctional axon guidance molecule during axon elongation and cell migration in the development of diencephalon and its connections. Mutations of Sema5A are linked to the etiology of some disorders and syndromes, as microdeletion of SEMA5A found in patients with autism spectrum and haploinsufficiency for SEMA5A in Cri-du-chat syndrome. We are lacking data on SEMA5A protein expression in human brain but according to the recent data (Allan Brain Institute), the transcripts of Sema5A gene are present in proliferative zones of the developing telencephalon in rodents at age E13.5 E15.5 and P14.
In the present study, we used in vitro functional essay and post-mortem protein expression analysis to reveal possible role of Sema5A in mammalian brain development. The neocortical explants from mouse embryo (E15) were cultivated 72h on Sema5a uniform or alternately presenting carpet (made of membrane homogenate of Sema5A transfected Human Embryonic Kidney 293 cells.)
Results of the in vitro study show that Sema5A promotes outgrowth and elongation of neocortical axons when uniformly presented on the carpet, but does not steer axons or modulate collateralization on Sema5A-alternating carpets. The expression of Sema5A protein revealed by immunohistochemical staining was more prominent at P15 than at P5 in the entire cortex with the clear regional differences in the expression. The most prominent change in expression was in somatosensory cortex suggesting a role of Sema5a in the development of the thalamocortical connections and the area in the observed early postnatal period. Further postmortem studies are needed to get a better understanding of Sema5A interaction with specific proteoglycans in the extracellular matrix, which subsequently would lead to a disclosure of the Sema5A role in the development of the telencephalon connectivity in health and disease.

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